Recent advances in molecular biology open the possibility to use formalin-preserved specimens stored in ichthyoplankton collections for population genetics studies. Nine DNA extraction techniques were tested on Engraulis mordax larvae preserved in buffered formalin. However, none of the DNA extracts resulted in the positive amplification of mitochondrial DNA (mtDNA) (NADH1, 16srRNA, 12srRNA and control region fractions). An experiment with different length-time exposure to formalin done with Cynoscion parvipinnis larvae allowed us to confirm the difficulty of amplifying mtDNA from larvae preserved in formalin for long time periods and the possibility of DNA extraction and amplification from short-term (less than 48 h) formalin-fixed marine fish larvae preserved in ethanol (70%). We discuss the possible influence of physical–chemical complexes associated with the duration of preservation to inhibition of amplification reactions.

INTRODUCTION

In the last 20 years, advances in molecular biology and medical research has led to extraction, amplification and sequencing of DNA from different tissues, including fossil amber, bones, minute larvae, leaves, mummies, individual hair follicles, dried blood, dung and a myriad of archival specimens, including formalin-fixed and ethanol-preserved specimens (Shiozawa et al., 1992; France and Kocher, 1996; Shedlock et al., 1997; Chase et al., 1998; Klanten et al., 2003).

Finding an appropriate DNA extraction technique will be very important for ichthyoplankton research because there are many collections that contain old series of specimens representing an invaluable source of information for evolutionary, phylogenetic and population genetic studies (Chase et al., 1998). We particularly chose northern anchovy larvae (Engraulis mordax GIRARD 1854) because of its ecological and economic significance in the Northeastern Pacific, and because there are several aspects of evolutionary history that need to be clarified. For instance, it is unknown whether the population that inhabits the Gulf of California is an extension of the southern population of the western coast of Baja California (Hammann and Cisneros, 1989) or is a resident subpopulation (Holmgren-Urba and Baumgartner, 1993).

We tested nine DNA extraction techniques, most of them proposed for formalin-fixed and ethanol-preserved tissues, to determine the efficacy of the techniques for DNA recovery and mitochondrial DNA (mtDNA) amplification from anchovy larvae that were fixed in buffered formalin (properly formalin preserved) for extended time periods.

METHOD

Larvae and adult samples of E. mordax were obtained from the central region of the Gulf of California. Larvae samples, collected during a survey cruise in March 1999, were fixed and preserved in 4% buffered formalin (neutralized to pH = 7.0 with sodium borate). The adult sample, used as a control, was obtained in a single cast in November 2001 and frozen in situ and afterwards preserved in ethanol (70%).

Engraulis mordax DNA from adults was extracted following Taggart et al. (Taggart et al., 1992) and was used to amplify by polymerase chain reaction (PCR) the following mtDNA fractions: NADH1 (1930 bp), 16srRNA (618 bp), 12srRNA (431 bp) and two fragments of the control region, one of 551 bp (control region II) and the other within this fragment of 176 bp (control region III).
(Table I). Amplifications were done in a Progene thermal cycler (Techne), in 25 μL reactions containing PCR buffer solution (1X), MgCl₂ (3.5 mM), dNTPs (80 μM, each), primers (0.48 μM, each), Taq polymerase (0.625 U) and distilled water.

The thermal conditions initially standardized in the control sample for the fractions NADH₁, 16sRNA and control region II were 2 min of denaturing at 94°C, 30 cycles of denaturing (94°C, 1 min), annealing (temperatures indicated in Table I, 1 min), and extension (72°C, 2 min) and a final extension (72°C, 4 min). The denaturing, annealing and extension steps for the amplification of 12sRNA and control region III fractions were 30–40 sec each. The PCR products of 16sRNA and control region II fractions were purified with the Qiagen Gel Extraction Kit and sequenced in an automatic sequencer (ABI Prism 310, PerkinElmer, Inc.).

Nine DNA extraction techniques, previously proposed to recover DNA from formalin-fixed ethanol-preserved specimens, were tested on formalin-fixed E. mordax larvae at least three times for each specimen. With each technique, DNA was extracted either from a single whole larva or from a combination of four whole larvae. The techniques were those of Heller et al. (Heller et al., 1992), Shiozawa et al. (Shiozawa et al., 1992) modified by France and Kocher (France and Kocher, 1996), Taggart et al. (Taggart et al., 1992), Shedlock et al. (Shedlock et al., 1997), Eckerman and Walsh (unpublished data), Jones and Avise (Jones and Avise, 1997), DNeasy Tissue Kit (Qiagen, Germany), DNeasy Tissue Kit modified by Chase et al. (Chase et al., 1998) and Coombs et al. (Coombs et al., 1999). Schematics for each procedure are given in Fig. 1. MtDNA fractions and amplification conditions used on formalin-fixed anchovy larvae samples were the same as those standardized in adults. DNA extracts and PCR products were revealed by electrophoresis in agarose gel (1%) and SybrGold staining. The quality and concentration of DNA extractions were evaluated by the proportion of optical density readings (OD₂₆₀/OD₂₈₀) in a Beckman DU-600 spectrophotometer and the Warburg-Christian algorithm, respectively.

### RESULTS

Five mtDNA fractions of E. mordax adults were amplified: NADH₁, 16sRNA, control region II, 12sRNA and control region III. The sequences of 16sRNA and control region II fractions were deposited at GenBank (accession numbers AY328468 and AY328469, respectively). High similarity values with sequences of two other engraulid species Engraulis japonicus (accession number AB040676) and Engraulis ringens (accession number AY184229), verified the identity of fractions reported here, discarding the possibility of false amplifications in the control sample from contamination or PCR artifacts.

Genomic DNA of formalin-fixed E. mordax larvae was visualized by techniques 3, 5 and 6; however, the DNA was very fragmented and of very low molecular weight (Fig. 2). Spectrophotometer readings showed negative values of concentration and OD₂₆₀/OD₂₈₀, indicating concentrations below the minimum required by the apparatus.

DNA amplification of all mtDNA fractions was tested with larval DNA extracts obtained with all techniques; nevertheless, none of them were positive. To test the hypothesis that the formalin preservation process and

### Table I: Forward (F) and Reverse (R) primers employed for amplification of several mitochondrial DNA regions of Engraulis mordax

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Primers</th>
<th>Annealing temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH1</td>
<td>F: 5'-ACCCCGCCTGGTTATCCAAAAACAT-3'</td>
<td>52</td>
<td>Cronin et al. (1993)</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GGTGATGGCCGATAGCTTA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16sRNA</td>
<td>F: 5'-GGCCGTGTATACAAAAACAT-3'</td>
<td>53</td>
<td>Palumbi et al. (1991)</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GGGTCTGAAACTCAGATACGT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control region II</td>
<td>F: 5'-ATTCTCTGCTTCTGTTTCTC-3'</td>
<td>56</td>
<td>This study*</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GCTCTCTGATGCAGTTGCTTTG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12s rRNA</td>
<td>F: 5'-AACGTGATGATGATCACCAGTT-3'</td>
<td>55</td>
<td>Lehman et al., 1996</td>
</tr>
<tr>
<td></td>
<td>R: 5'-AAAGGGACGGGCGATGTTG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control region III</td>
<td>F: 5'-ATTCTGCTTGCTTCTGTTTCT-3'</td>
<td>57</td>
<td>This study*</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CCAATGCAAGTGAAAGGTG-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Primers were designed for Engraulis mordax by means of Primer 3 software (Rozen and Skaltsky, 2000) that was based on the mitochondrial DNA (mtDNA) sequence from Engraulis japonicus (Inoue et al., 2001).

b Primers were designed from Control region II sequence of E. mordax.
Fig. 1. Flow chart of nine DNA extraction techniques tested. Symbols used: \( \square \), formalin elimination; \( \bigcirc \), digestion; \( \square \), additional quantities; \( \bigcirc \), purification; \( \triangle \), precipitation; \( \square \), washing; \( \triangledown \), sonication; \( \square \), preservation; \( \bigtriangleup \), ebullition; \( \bigtriangleup \), unknown process; PBS, phosphate-buffered saline solution (Sambrook et al., 1989); ATL, AL, AW1, AW2, AE buffers: manufacturer solutions (Qiagen).
its duration were responsible for the negative results, we did an experiment of DNA extraction from fish larvae fixed in formalin, but preserved in ethanol. Because no E. mordax larvae were available, the experiment was done with hatchery-reared drum larvae (Cynoscion parvipinnis) at the 3-d stage of development. Experimental design consisted in five treatments in buffered formalin (4%) with five larvae per treatment. Each treatment consisted in fixing larvae for different lengths of time (5 min, 4 h, 24 h, 48 h, and 8 months) and then preserving the larvae in 70% ethanol. The control group was fixed and preserved in 70% ethanol. Analysis was done at the same time, therefore all larvae stayed preserved for 8 months. DNA extraction was done for each individual larva using the DNeasy Tissue Kit and was used to amplify 16srRNA. Positive and negative controls were used in all PCR reactions.

We observed a gradual degradation in DNA extracts that increased with the length of exposure time to formalin; the effects on DNA quality were most evident after 4 h of formalin fixation (Fig 3a and b). Successful mtDNA amplification was possible for all treatments, with the exception of larvae maintained in formalin for

Fig. 2. Extractions of DNA from Engraulis mordax larvae with different techniques. Each technique includes two lanes that correspond to the extraction of one larva (left) and grouping four larvae (right). *, successful extraction.

Fig. 3. DNA extracts (a, b) and 16srRNA amplification products (c, d) from Cynoscion parvipinnis larvae. AE, Engraulis mordax adult preserved in ethanol (70%); NC, negative control; L, DNA ladder.
8 months (Fig. 3c and d). PCR products that corresponded to the expected 16srRNA fragment (618 bp) were obtained. Sequences of these products matched with a C. parvipinnis sequence published in the GenBank (access number AY958648). The comparisons among sequences under different treatments (ethanol, 5 min, 4 h, 24 h, and 48 h) showed no mutations within the fragment (data not shown).

DISCUSSION

At the molecular level, DNA is packed into chromosomes in a tight structure with the help of specialized proteins. When live tissues are fixed with formalin, it penetrates the tissue and causes cross-linking of all proteins in the chromosomes (Ren et al., 2000). Some researchers believe that cross-linking occurs when the unpaired electrons of the oxygen atom in formaldehyde (CH2O) reacts with primary amines (−NH2) (France and Kocher, 1996). The cross-linking effect is stronger when fixation is extended, causing a stable matrix that tenses the tissue, preserving the cellular structure and preventing release of double helix DNA from the complex (Chaw et al., 1980; Chang and Loew, 1993; Chatigny, 2000; Ren et al., 2000).

In formalin-preserved anchovy larvae, the extracted DNA with techniques 3, 5 and 6 was very fragmented, indicating that cross-linking does not inhibit the extraction procedures of DNA. Successful extraction techniques shared the following characteristics: (i) elimination of formalin remnants before digestion to protect protelysis effectiveness (Shedlock et al., 1997) by the sample incubation in buffer solution volumes (Shiozawa et al., 1992) or previous washes in phosphate-buffered saline (PBS) solution (DNeasy Tissue Kit), (ii) use of proteinase-K (Qiagen) to assure complete proteolysis and release of nucleic acids, (iii) diminution of loss of DNA during the organic purification process (Chase et al., 1998) by decreasing reaction volumes (France and Kocher, 1996) or by using a membrane (DNeasy Tissue Kit) and (iv) DNA extracts were diluted with minimum volumes of buffer solution to increase their concentration.

Despite the recommendation of Shedlock et al. (Shedlock et al., 1997) to use primers that amplify fragments of low molecular weights ranging from 100 to 200 bp, like the control region III fraction, we were not able to amplify mtDNA from formalin-preserved E. mordax larvae. The most important finding from this negative effect is the fixation time with formalin. Shiozawa et al. (1992), France and Kocher (1996), Shedlock et al. (1997), Wirgin et al. (1997) and Klanten et al. (2003) successfully amplified mtDNA fragments because, in those studies, the tissue was fixed in formalin (4–10%) for 24–48 h and then preserved in alcohol (70–80%) for various time periods. In our study, the process employed with E. mordax larvae was to fixate and preserve in formalin for more than 3 years (properly formalin preservation). These results suggest that after a long time in formalin preservation, it is unlikely that mtDNA fragments can be recovered by amplification. These results are confirmed by our experiments with C. parvipinnis larvae, in which mtDNA could be amplified on formalin-fixed, ethanol-preserved larvae over short time periods, but not on longer periods of preservation in formalin (i.e. 8 months).

Understanding DNA–formalin interactions still remains incomplete. Earlier, we mentioned that fixation in formalin causes cross-linking (Ren et al., 2000). If this is the case, the cross-linking process could create highly fragmented DNA in E. mordax larvae that was impossible to amplify. It is also possible that during DNA extraction, the DNA-protein bonds had not been completely eliminated, therefore inhibiting the PCR reaction. Protein remnants and other compounds decrease the quality and quantity of DNA that can be amplified (McPherson and Möller, 2000; Ren et al., 2000).

To extract good quality DNA from marine fish larvae for PCR amplification and carry out studies about genetic diversity and structure, we strongly suggest that fixation in buffered formalin should be less than 48 h and followed by preservation in ethanol (70%). For the DNA extraction process, we found the DNeasy Tissue Kit practical and successful. For those marine fish larvae that remain preserved in formalin in ichthyologic collections, it will still be necessary to find other purification processes that optimize the elimination of protein complexes from extracted DNA and try to amplify even smaller DNA fragments, such as microsatellites.

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